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The Prodrug Activator EtaA from *Mycobacterium tuberculosis* Is a Baeyer-Villiger Monooxygenase*

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EtaA is a newly identified FAD-containing monooxygenase that is responsible for activation of several thioamide prodrugs in *Mycobacterium tuberculosis*. It was found that purified EtaA displays a remarkably low activity with the antitubercular prodrug ethionamide. Hinted by the presence of a Baeyer-Villiger monooxygenase sequence motif in the EtaA sequence, we have been able to identify a large number of novel EtaA substrates. It was discovered that the enzyme converts a wide range of ketones to the corresponding esters or lactones via a Baeyer-Villiger reaction, indicating that EtaA represents a Baeyer-Villiger monooxygenase. With the exception of aromatic ketones (phenylacetone and benzylacetone), long-chain ketones (*e.g.* 2-hexanone and 2-dodecanone) also are converted. EtaA is also able to catalyze enantioselective sulfoxidation of methyl-*p*-tolylsulfide. Conversion of all of the identified substrates is relatively slow with typical k_{cat} values of around 0.02 s^{-1}. The best substrate identified so far is phenylacetone (K_m = 61 μM, k_{cat} = 0.017 s^{-1}). Redox monitoring of the flavin cofactor during turnover of phenylacetone indicates that a step in the reductive half-reaction is limiting the rate of catalysis. Intriguingly, EtaA activity could be increased by one order of magnitude by adding bovine serum albumin. This reactivity and substrate acceptance-profiling study provides valuable information concerning this newly identified prodrug activator from *M. tuberculosis*.

Tuberculosis is the world's leading cause of death from a single infectious organism. Standard tuberculosis chemotherapy consists of a lengthy treatment with at least two so-called first-line drugs, *e.g.* isoniazid and rifampicin. Unfortunately, an increasing number of tuberculosis cases are encountered that cannot be treated because of resistance to one or more of these drugs (1, 2). The last resort for combating such (multi)-drug-resistant mycobacterial infections relies mainly on the action of second-line antitubercular drugs. For this reason, several thioamide drugs have been successfully employed during the last decades (Fig. 1). However, as for other antitubercular agents, more and more infections are encountered that are also resistant to thioamide-based treatment. Despite their widespread use, the mode of action of thioamide antibiotics as well as the mechanism by which mycobacteria acquire resistance against these drugs has remained obscure.

A few years ago a gene from *Mycobacterium tuberculosis* was identified that is responsible for the antitubercular effect of ethionamide, which was named *etaA* (3, 4). Ethionamide was found to be activated by the corresponding protein EtaA, after which the activated product exerts a lethal effect by interacting with a final target. The activating role of EtaA was confirmed by overexpression of the prodrug activator in *Mycobacterium smegmatis*, resulting in ethionamide-hypersensitive mycobacteria. Clinically observed resistance toward the prodrug could be associated with *etaA* gene mutations resulting in functional impairment of EtaA. A thorough analysis of ethionamide-resistant *M. tuberculosis* isolates has revealed cross-resistance to two other widely used thiocarbamide-containing drugs, thiacetazone and thiocarlide (see Fig. 1) (3). In addition, it is very likely that the antitubercular effect of prothionamide can also be linked to activation by EtaA because it differs from ethionamide by only one methyl group. This indicates that EtaA displays an exceptional broad prodrug acceptance. Furthermore, because ethionamide is also used to treat leprosy, a similar activating enzyme is expected to be present in this related pathogenic *Mycobacterium leprae*. This is in line with the observation that, similar to *M. tuberculosis*, cross-resistance among above-mentioned thioamides has been observed for *M. leprae* (5). Genome analysis indeed shows that, despite a dramatically reduced genome size, an *etaA* gene ortholog has been conserved in the *M. leprae* genome (6, 7).

Recently, the primary final target of EtaA-activated ethionamide has been identified to be InhA, an enoyl-acyl carrier protein reductase involved in fatty acid synthesis (8). InhA is also the final target for another well known antitubercular prodrug, isoniazid. Although sharing the same final target, the prodrugs ethionamide and isoniazid are specifically activated by two unrelated redox enzymes. While isoniazid is activated by a heme-containing catalase-peroxidase (KatG) (9), it has recently been shown that EtaA is a flavin-containing monooxygenase (10). EtaA-mediated conversion of ethionamide results in the formation of the corresponding sulfoxide product. This sulfoxide product does not represent the cytotoxic species but rather has to be activated in a subsequent reaction. This second activation step was also found to be catalyzed by EtaA yielding 2-ethyl-4-amidopyridine as final product (10). However, this amide has no antitubercular activity, indicating that the key toxic species is formed as an unstable reactive product intermediate (3). It has been suggested that the initial sulfinate product formed after two consecutive EtaA-mediated sulfoxidations of ethionamide decomposes to form a labile toxic intermediate. This intermediate would resemble activated isoniazid inhibiting the final target InhA by forming a covalently modi-

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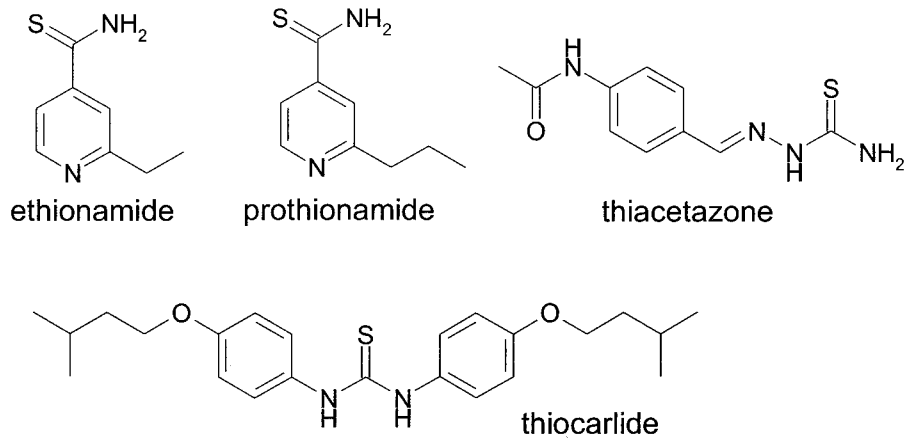


FIG. 1. **Structural formulas of some thiocarbamide-containing antituberculosis drugs.**

fied NADH derivative in the active site of InhA (11). Nevertheless, it has been observed that the effect of activated ethionamide on InhA activity differs from isoniazid-based inhibition as inhibition cannot be prevented by the addition of NADH or NAD^+ (12). This finding suggests a different mode of action of the activated product. Furthermore, it has been found that the final target for other antitubercular thioamides may differ for each thioamide prodrug. Different from ethionamide and isoniazid, thiocarlide and related thioamide compounds specifically affect the synthesis of short-chain fatty acids (13).

A sequence homology search using the EtaA sequence revealed significant homology with several flavin-containing monooxygenases. Furthermore, the presence of a newly identified sequence fingerprint (F*X*G*XXX*H*XXX*W(P/D)) suggests that EtaA is a flavin-containing Baeyer-Villiger monooxygenase (BVMO)[1] (14). In fact, a search in the protein sequence data base revealed that the closest homolog (24% sequence identity) with known activity is 4-hydroxyacetophenone monooxygenase. This BVMO has recently been cloned and expressed in our laboratory (15) and prompted us to study the substrate specificity and reactivity of the recently identified prodrug activator EtaA.

EXPERIMENTAL PROCEDURES

Chemicals—Restriction enzymes, NADH, NADPH, glucose oxidase (grade II), and catalase, were obtained from Roche Applied Science. Ketones were purchased from Acros, Aldrich, and Lancaster. $NADP^+$, 3-acetyl-$NADP^+$, and 3-amino-$NADP^+$ were from Fluka. All of the other chemicals were of commercially available analytical grade. DNA samples were purified using the QIAQuick gel and purification kit from Qiagen. *Escherichia coli* TOP10-competent cells and the pBAD/*myc*-HisA vector were obtained from Invitrogen. Purified recombinant 4-hydroxyacetophenone monooxygenase was obtained as described previously (15).

Expression and Purification of Recombinant EtaA—The *etaA* gene was amplified using pMH29-containing *etaA* as template (kindly provided by C. E. Barry, III and A. E. DeBarber, National Institutes of Health) and the following two primers: PETA1 (5′-GGCACGGCATATGACCGAGCACCTCGACGTTGTC-3′, NdeI site is shown *underlined*) and PETA2 (5′-GGCACGGAAGCTTAACCCCCACCGGGGCAGGCCTTTTGG-3′, HindIII site is shown *underlined*). For expression, a pBAD-/*myc*-HisA (Invitrogen) derived expression vector, pBADNK, was used in which the original NdeI sites have been removed, whereas the NcoI site has been replaced by a NdeI site (16). After amplification, the gene was isolated from gel, digested with NdeI and HindIII, and ligated behind the *araBAD* promoter of the NdeI/HindIII-digested pBADNK-yielding pBETA1. Expression using pBETA1 results in the production of EtaA containing 25 additional C-terminal residues (c-*myc* epitope and His_6). Expression of native EtaA was achieved by introducing a stop codon in pBETA1 using the primers PETA3 (5′-GCCCCGGTGGGGGTT**TGA**AAGCTTGGGCCCGAAC-3′, HindIII site is shown *underlined*) and PETA4 (5′-GTTCGGGCCCAAGCTT**TCA**AACCCCCACCGGGGC-3′, HindIII site is shown *underlined*) resulting in pBETA2. For expression, *E. coli* TOP10 cells were transformed with pBETA1 or pBETA2 and grown in LB medium supplemented with 100 μg/ml ampicillin and 0.02% (w/v) arabinose at a temperature of 25 °C.

His-tagged EtaA was purified using the following procedure. Cells from a 2-liter culture were harvested by centrifugation and resuspended in TMAG (50 mM Tris, 100 mM KCl, 1 mM β-mercaptoethanol, 1 mM NaN_3, 10% v/v glycerol, pH 7.5, containing 1% (v/v) Triton X-100). After sonication and centrifugation, the resulting supernatant was added to nickel-agarose affinity column material with a capacity of 50 mg. By means of end-over-end rotation, the column material was resuspended with the extract and incubated for 1 h at 4 °C. After incubation, the column material was washed by gravitational flow. Upon washing the column with 10 column volumes of TMAG and 10 column volumes of TMAG containing 20 mM imidazole, the enzyme was washed from the column with TMAG containing 400 mM imidazole. To remove the imidazole, the collected enzyme fraction was applied to a preequilibrated Hi-prep 26/10-desalting column (Amersham Biosciences). Finally, the enzyme was concentrated by using an Amicon ultrafiltration unit equipped with a 100-kDa cut-off filter and stored at −80 °C.

Native EtaA was purified using three columns. The harvested cells were resuspended in buffer (20 mM potassium phosphate, 1 mM β-mercaptoethanol, 1 mM NaN_3, 10% v/v glycerol, pH 7.5), sonicated, and centrifuged. In the first column step, the extract was applied onto a Q-Sepharose column. The enzyme could be eluted using 250 mM KCl. In the second step, the collected enzyme fraction was applied onto a ceramic hydroxyapatite column (2.5 × 12 cm, Type I, particle size 20 μm, Bio-Rad) equilibrated with 20 mM potassium phosphate, pH 7.0. The enzyme was eluted using a gradient of 20–300 mM potassium phosphate. Active fractions were concentrated by ultrafiltration and applied to a 26/60 Superdex 200 column (Amersham Biosciences). Again, active fractions were pooled and concentrated by ultrafiltration and subsequently stored at −80 °C.

Analytical Methods—Analytical size-exclusion chromatography was performed using a Superdex 200 HR 10/30 column (Amersham Biosciences) using TMAG with or without 0.5 mg/ml BSA. Aliquots of 100 μl were loaded on the column and eluted at a flow rate of 0.8 ml/min. Apparent molecular masses were determined using a calibration curve made with standards from the molecular mass marker kit from Bio-Rad: thyroglobulin (670 kDa); bovine γ globulin (158 kDa); chicken ovalbumin (44 kDa); equine myoglobin (17 kDa); and vitamin B12 (1.35 kDa).

Enzyme activity with all of the tested ketones was determined spectrophotometrically by monitoring the decrease of NADPH at 340 nm ($\epsilon_{340\ nm} = 6.22\ mM^{-1}\ cm^{-1}$). Conversion of ethionamide was measured by monitoring the formation of the ethionamide-oxide product at 400 nm ($\epsilon_{400\ nm} = 1.0\ mM^{-1}\ cm^{-1}$). Reaction mixtures (1.0 ml) typically contained 50 mM Tris/HCl, pH 8.5, 0.2 mM NADPH, and 1.0 μM enzyme. For determining the K_m for NADPH, 1.0 mM phenylacetone was used as substrate. For measuring the effect of additives, 0.5 mM phenylacetone and 0.2 mM NADPH were used. All of the kinetic measurements were performed at 25 °C using air-saturated buffers.

The redox potential of EtaA was determined by the method of Massey (17). Enzyme (5 μM) in the presence of 2 μM methyl viologen, 5 μM phenosafranin ($E_m = -252$ mV), 200 μM xanthine, and 10% (w/v)

[1] The abbreviations used are: BVMO, Baeyer-Villiger monooxygenase; EtaA, ethionamide monooxygenase; BSA, bovine serum albumin; GC-MS, gas chromatography-mass spectrometry; FAD, flavin adenine dinucleotide.

glycerol in 50 mM Tris/HCl, pH 7.5, was made anaerobic in a cuvette by flushing with nitrogen. Reduction was initiated by adding 0.5 μg/ml xanthine oxidase and subsequently monitored using a PerkinElmer Lambda Bio 40 spectrophotometer (at 25 °C). Spectra (300–700 nm) were recorded every minute until the reduction of both dye and enzyme was complete (~1.5 h). Reduction of the dye was measured at 520 nm, whereas flavin reduction could be followed at 404 nm.

Product Identification—For product identification, samples (1.0 ml) containing 5 mM of substrate, 2.5 mM NADPH, and 2 μM EtaA were incubated for 24 h at 25 °C. The reaction was quenched by adding 1.0 ml of ethyl acetate containing 1.0 mM hexadecane. The ethyl acetate layer was collected and dried over $MgSO_4$ and subsequently analyzed by GC-MS. GC-MS analysis was performed on a Hewlett Packard HP 5890 series II gas chromatograph and a Hewlett Packard HP 5971 mass spectrometer equipped with an HP-5 column. Samples (1 μl) were injected without derivatization and using the following temperature program: a gradient from 50 to 150 °C in 10 min, 4-min isothermal at 150 °C followed by a gradient from 150 to 300 °C in 3 min, and finally 12-min isothermal at 300 °C. The enantioselective sulfoxidation of methyl-*p*-tolylsulfide was monitored by GC using a Chiraldesc G-TA column (30 m × 0.25 mm) (16).

RESULTS

Purification and Spectral Characterization of Recombinant EtaA—EtaA containing a His_6 and c-*myc* epitope tag at the C terminus was purified from cell extracts in the presence of Triton X-100. From 1 liter of culture, ~15 mg of recombinant protein could be purified. The purified enzyme migrated as a single band in SDS-PAGE corresponding to a mass of ~56 kDa. This value is close to the theoretical mass of the recombinant protein (58,216 Da). Elimination of the detergent from the purification buffer resulted in very low yields (<10% compared with the addition of 0.1% Triton X-100), suggesting that the enzyme is membrane-associated. Solubilization of EtaA by Triton X-100 has been observed previously (10). However, no clear hydrophobic segments could be identified in the EtaA sequence that could serve as a membrane anchor. The EtaA sequence shows homology with a number of other flavin-containing monooxygenases that are all known to be monomeric or dimeric soluble proteins (14). Nonetheless, it was found that EtaA tends to form oligomers as evidenced by gel filtration experiments. Gel filtration experiments performed in this study showed that the enzyme exists as a mixture of relatively large oligomers ranging from 200 to 600 kDa.

Compared with His-tagged EtaA, purified native EtaA displayed similar hydrodynamic properties and showed the same activity toward ethionamide (see below). As purification of His-tagged enzyme is very efficient, this EtaA variant has been used for all of the experiments described below.

EtaA was purified as a brightly yellow protein indicating that it contains a tightly bound flavin cofactor, which has previously been identified as FAD (10). Spectral analysis of the purified recombinant enzyme showed a typical flavin spectrum displaying absorbance maxima at around 367 and 451 nm (Fig. 2). The ratio in absorbance at 280 and 451 nm for the purified protein was typically 16. This ratio was found to be the same for all of the oligomeric species observed upon gel filtration, indicating that flavin content is not dependent on the oligomeric state. Unfolding of the enzyme by 0.05% SDS resulted in a rapid release of the flavin cofactor allowing quantification of the released cofactor. Combining the determined cofactor and protein subunit concentrations showed that the purified enzyme contains stoichiometric (1:1) amounts of the flavin cofactor. The molar extinction coefficient for EtaA could be calculated using the known value of free FAD ($\epsilon_{451\ nm\ EtaA}$ = 11,600 $M^{-1}\ cm^{-1}$).

Catalytic Properties of EtaA—To probe whether all of the EtaA-bound FAD participates in catalysis, the enzyme was mixed anaerobically with an excess of NADPH (Fig. 2). It was found that the enzyme was slowly reduced by the nicotinamide

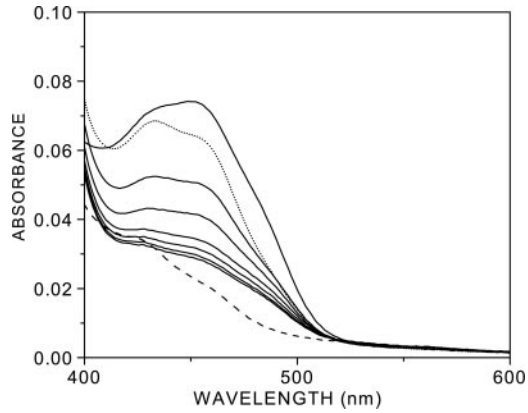


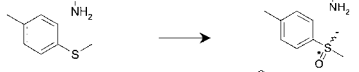
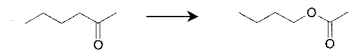
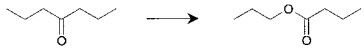
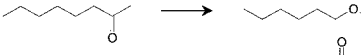
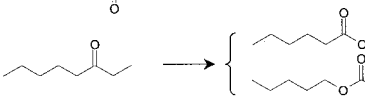
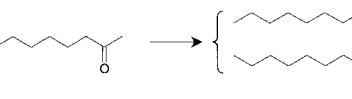
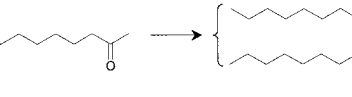
FIG. 2. **Spectral changes observed upon reduction of EtaA.** EtaA (6.3 μM) was anaerobically reduced by 150 μM NADPH in 50 mM Tris/HCl, pH 8.5. Spectra (from *top* to *bottom*) were recorded at t = 0, 5, 10, 15, 20, and 30 min. A spectrum of fully reduced EtaA (—) was obtained by adding dithionite. Aerobic incubation of EtaA with 440 μM NADPH and 430 μM phenylacetone shows that the enzyme is mainly in the oxidized form during steady-state turnover (·····, spectra obtained after 2-min incubation).

coenzyme. Comparing the spectrum reached after 30-min incubation with NADPH with the spectrum obtained by reduction with dithionite shows that the enzyme is almost fully reduced. The fact that the enzyme is able to bind and react with NADPH indicates that the enzyme is folded and catalytically competent. No significant flavin reduction could be measured when using NADH. This is in line with the observation that all of the characterized sequence-related monooxygenases are highly specific for NADPH (14). Aerobic incubations with NADPH revealed that the enzyme catalyzes a slow oxidation of NADPH (0.0045 s^{-1}) generating hydrogen peroxide. A low NADPH oxidase activity has also been observed with other BVMOs (15, 19).

Using ethionamide and NADPH as substrates, a relatively slow sulfoxidation of the thioamide could be observed, confirming the activating role of EtaA. Conversion of ethionamide could be directly monitored by measuring the absorbance of the sulfoxide product at 400 nm (10). Oxygen concentration was not limiting the rate of ethionamide conversion because using oxygen-saturated solutions did not increase the conversion rate. The determined steady-state kinetic parameters of EtaA for ethionamide oxidation suggest that ethionamide is a remarkably poor substrate for the prodrug activator displaying a relatively low k_{cat} value of 0.027 s^{-1} and a K_m of 340 μM (Table I). Such a low rate of conversion does not seem to be of physiological importance because conversion rates (k_{cat}) for FAD-containing monooxygenases typically range from 1 to 30 s^{-1} (18, 20). In fact, it is difficult to envisage that such an inefficient prodrug activation can initiate the bactericidal effect observed in mycobacteria. The enzyme was found to be efficient in NADPH recognition displaying a K_m of 10 μM using ethionamide as oxygenating substrate. This value is similar to those found for other sequence related-monooxygenases (18).

It is known that flavin-containing monooxygenases can with the exception of catalyzing oxygenations also generate hydrogen peroxide. In fact, the addition of certain effector molecules can accelerate such a NADPH oxidase activity. For example, the FAD-containing 2-hydroxybiphenyl 3-monooxygenase from *Pseudomonas azelaica* HBP1 uses, depending on the substrate, only a small part of all consumed NADPH molecules to hydroxylate the substrate, whereas all of the other electron equivalents are used for hydrogen peroxide formation (21). Because hydrogen peroxide is a potent oxidative agent, it was imperative to test whether the observed sulfoxidation reactions catalyzed by EtaA are truly enzyme-mediated. For this reason, we

TABLE I
Identified EtaA substrates and corresponding products
In case of formation of two products the ratio in product formation as estimated by GC analysis is indicated.

Substrate	Product
ethionamide [a]	
methyl-*p*-tolylsulfide	
phenylacetone	
benzylacetone	
bicyclohept-2-en-6-one	
2-hexanone	
2-heptanone	
4-heptanone	
2-octanone	
3-octanone	 33 67
2-decanone	 3 97
2-dodecanone	 89 11

[a] Product has been identified by Vannelli *et al.* (10).

examined the EtaA-mediated conversion of methyl-*p*-tolylsulfide, a sulfide that is readily accepted by other flavin-dependent monooxygenases as substrate (18). It was found that EtaA catalyzes an enantioselective oxygenation of this sulfide, yielding mainly the S-sulfoxide (55% enantiomeric excess). The addition of catalase to the incubation mixture did not influence the observed enantioselectivity. These results indicate that the sulfide is oxidized in the active site of EtaA by reacting with the electrophilic hydroperoxy form of the flavin cofactor (18), confirming a direct role of EtaA in sulfide oxidation.

EtaA Catalyzes Baeyer-Villiger Reactions—Hinted by the presence of a BVMO identifying sequence motif in the EtaA sequence (14), we tested a range of ketones that have been shown to be converted by BVMOs. It was found that several typical BVMO substrates (cyclohexanone, acetophenone, and 4-hydroxyacetophenone) were not accepted by EtaA. Incubations with these ketones did not increase consumption of NADPH nor could the expected lactone or ester products be detected by GC-MS analysis. However, although the addition of bicyclohept-2-en-6-one did not increase the rate of NADPH oxidation, a small amount (~1% conversion) of a Baeyer-Villiger oxidation product, 3-oxabicyclo[3.3.0]oct-6-en-2-one, could be detected. This prompted us to test more aliphatic ketones. By GC-MS analysis, it was found that a surprisingly broad range of ketones was converted by EtaA (Table I). Besides several long-chain alkanones ranging from 2-hexanone to 2-dodecanone, phenyl substituent-bearing ketones also are converted via a Baeyer-Villiger reaction into the corresponding esters. With the exception of the long-chain alkanones 2-octanone, 2-decanone, and 2-dodecanone, conversion of all of the ketones resulted in the formation of the expected ester product. In the case of 3-octanone, 2-decanone, and 2-dodecanone, some formation of the chemically less favored ester products could also be detected. By this, EtaA-mediated oxidation obeys the stereochemical rule of Baeyer-Villiger oxidation reactions where the oxygen is typically inserted by migration of the most substituted alkyl chain. For some of the identified substrates, the steady-state parameters were determined (Table II). Remarkably, all of the measured k_{cat} values are in the same range as determined for ethionamide. Only the K_m values varied to a significant extent, revealing that phenylacetone is the best substrate found so far.

TABLE II
Steady-state kinetic parameters of EtaA

Substrate	K_M	k_{cat}	FMT[a]
	μM	s^{-1}	%
Ethionamide	340	0.027	88
Phenylacetone	61	0.017	90
Benzylacetone	520	0.021	91
2-Dodecanone	200	0.023	94

[a] The flavin-monitored turnover (FMT) value indicates the percentage of oxidized cofactor during turnover.

The similar k_{cat} values found for all of the tested substrates suggest that the rate of oxygenation is determined by a shared slow event along the catalytic cycle. By determining the redox state of the enzyme during turnover, it can be established which half-reaction comprises this rate-limiting event. For these flavin-monitored turnover experiments, the absorbance at 440 nm was taken as the indicator for the presence of oxidized enzyme. By incubating the enzyme with saturating amounts of substrate, the redox state of EtaA during conversion of ethionamide, benzylacetone, phenylacetone, and 2-dodecanone was determined. For all of the tested substrates, it was found that the enzyme is mainly present in the oxidized state, indicating that a catalytic step in the reductive half-reaction is limiting the rate of catalysis.

During the reductive half-reaction, a hydride is transferred from the NADPH coenzyme to the flavin cofactor. This redox reaction will only proceed when the redox potential of the electron acceptor, the flavin cofactor, is higher relative to the redox potential of NADPH (−320 mV). Using the method described by Massey (17), the redox potential of EtaA was determined. During reduction of EtaA, no flavin radical intermediate could be observed, indicating that the reaction involves a 2-electron reduction. For determining the redox potential, phenosafranin (E_m = −252 mV) could be used as reference because reduction of this dye almost coincides with reduction of EtaA. Using the difference between the $\log(^{ox}_{red})$ values of the dye and EtaA during reduction, the redox potential for EtaA could be estimated: −245 ± 3 mV. This value is somewhat lower then the value for free FAD (-207 mV) and similar to values reported for other flavin-dependent monooxygenases (17, 22, 23). The measured redox potential is also in the expected range to facilitate reduction of the FAD cofactor by NADPH.

Identification of EtaA Activators and Inhibitors—Because the activity of EtaA with all of the uncovered substrates is very low, several compounds were tested for their activating or inhibiting effect (Table III). Because the enzyme appears to be membrane-associated (10), the influence of several detergents on activity was investigated. The addition of all of the tested detergents only resulted in decreased activities. For sequence related eukaryotic flavin-containing monooxygenases, specific activators have been found (24, 25). However, the tested compounds, *n*-octylamine and reduced glutathione, did not significantly affect EtaA activity. A striking effect was found when using bovine serum albumin as additive. The addition of this

TABLE III
The effect of additives on EtaA activity

Compound	Concentration	Activity
		%
		100
Triton X-100	0.1 %	65
Tween 20	0.1 %	20
Laurylsarcosine	0.1 %	0
Reduced glutathione	10 mM	60
n-Octylamine	5 mM	110
Bovine serum albumin	2 mg/ml	930
Diphenyleneiodonium	0.5 mM	100
Coenzyme analogs		
$NADP^+$	0.5 mM	86
NAD^+	0.5 mM	86
AMP	0.5 mM	80
3-Acetyl-$NADP^+$	0.5 mM	50
3-Amino-$NADP^+$	0.05 mM	26

protein increased the activity by one order of magnitude (Table III). By varying BSA concentrations, it was found that already at an equimolar concentration of BSA, EtaA activity was significantly increased (Fig. 3). Kinetic analysis using phenylacetone as substrate revealed a 15-fold increase in k_{cat} while the K_m value also significantly increased when 6 μM BSA was added (k_{cat} = 0.26 s^{-1}, K_m = 280 μM). Monitoring the flavin cofactor during conversion of phenylacetone revealed that the addition of bovine serum albumin increases the rate of flavin reduction. While in the absence of bovine serum albumin, the flavin is mainly in the oxidized state (Table II) and the reductive and oxidative half-reactions are almost balanced when bovine serum albumin is added (56% of the flavin is oxidized during catalysis). An increased rate of reduction is in line with the observed increase in k_{cat} and K_m values when a similar catalytic mechanism is operative as found for related flavin-containing monooxygenases (24).

To probe the effect of BSA on the oligomerization behavior of EtaA, a gel filtration experiment was performed using 6 μM BSA in the elution buffer. It was found that BSA did not affect the elution pattern because again a distribution of oligomers ranging for tetramers to decamers was observed. Apparently, the activating effect of BSA does not involve dissociation of oligomeric EtaA. These results suggest that EtaA is activated *in vivo* by protein-protein or interactions with other cellular components. Although bovine serum albumin is frequently used to stabilize enzymes in solution, an activating effect by this protein has been rarely reported. Interestingly, an activating effect by bovine serum albumin has also been observed with another BVMO, which acts on similar aliphatic substrates (26).

Besides exploring the effect of potential activators, a known flavoprotein inhibitor, diphenyleneiodonium, was also tested. However, no inhibitory effect was detected, which might indicate that the active site is not accessible for this relatively bulky compound (27). For the well studied BVMO, cyclohexanone monooxygenase, it is known that $NADP^+$ is an effective competitive inhibitor (22). The affinity of EtaA for $NADP^+$ and other coenzyme analogs was also probed (Table III). Both $NADP^+$ and NAD^+ were found to be poor inhibitors. $NADP^+$ analogs differing in the nicotinamide moiety (3-acetyl-$NADP^+$ and 3-amino-$NADP^+$) showed significant inhibition. 3-Amino-$NADP^+$ had a dramatic effect on activity as only 26% of the normal activity was measured with a relatively low inhibitor concentration (50 μM). This indicates that 3-amino-$NADP^+$ can bind tightly to the NADPH binding pocket while the reaction product $NADP^+$ cannot. Because the binding of 3-amino-$NADP^+$ might mimic the binding of NADPH close to the flavin cofactor, the redox potential of EtaA in the presence of 50 μM 3-amino-$NADP^+$ was measured. It was found that binding of this inhibitor results in a small upward shift of 11 mV: E_m = −234 ± 3 mV.

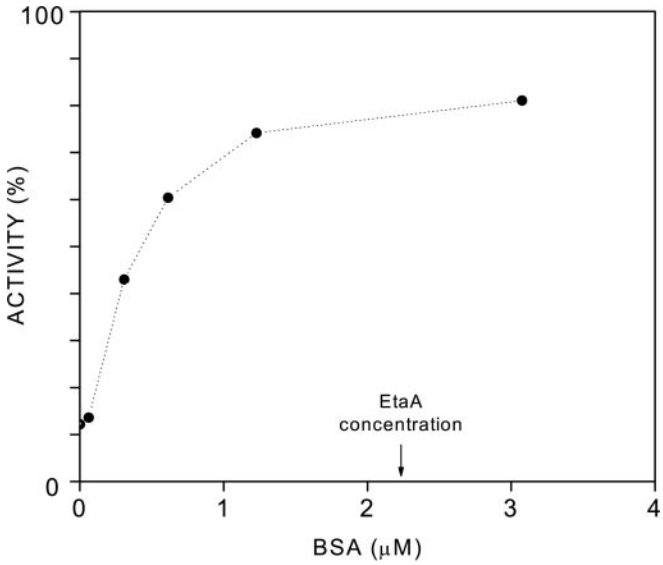


FIG. 3. **Activation of EtaA by BSA.** The activity of EtaA (2.2 μM) was measured in the presence of varying concentrations of BSA (0–3.1 μM) using 150 μM NADPH and 500 μM phenylacetone in 50 mM Tris/HCl, pH 8.5.

Activation of Ethionamide by 4-Hydroxyacetophenone Monooxygenase—As mentioned above, EtaA shares significant sequence identity with a bacterial BVMO, 4-hydroxyacetophenone monooxygenase. To check whether the sequence homology also extends to shared catalytic properties, the activity of 4-hydroxyacetophenone monooxygenase with ethionamide was tested. It was found that the enzyme rapidly converts ethionamide into the corresponding sulfoxide (k_{cat} = 1.1 s^{-1}, K_m = 330 μM). Similar to EtaA (10), 4-hydroxyacetophenone monooxygenase was also found to perform the subsequent oxidation of the formed sulfoxide since prolonged incubations resulted in the loss of absorbance at 400 nm. By comparing the steady-state kinetic parameters for ethionamide and NADPH for both bacterial monooxygenases, it can be concluded that 4-hydroxyacetophenone monooxygenase is very efficient in oxidizing ethionamide. In fact, the corresponding k_{cat} value is in the same range as found for 4-hydroxyacetophenone, its physiological substrate (15). Similar to EtaA, 4-hydroxyacetophenone monooxygenase is also able to sulfoxidize methyl-*p*-tolylsulfide forming the *S*-sulfoxide in high enantiomeric excess (>99%) (16). No activity with phenylacetone could be detected, indicating that the substrate profiles of both monooxygenases are not fully overlapping. Nevertheless, the relatively high activity of 4-hydroxyacetophenone monooxygenase with ethionamide indicates that this monooxygenase represents a convenient model enzyme to study the mechanism of ethionamide activation.

DISCUSSION

For decades, thioamides have been used to treat mycobacterial infections. Recently, it has been established that in *M. tuberculosis*, the toxicity of the most commonly used thioamide, ethionamide, depends on activation by a specific prodrug activator (3, 4). This activator, EtaA, represents a NADPH-specific FAD-containing monooxygenase (10). Sequence analysis suggested that the monooxygenase is member of a newly identified family of BVMOs (14). In this study, we have been able to demonstrate that EtaA is indeed able to perform Baeyer-Villiger oxidations reactions. The ability to catalyze such atypical oxygenation reactions and the relatively

high degree of sequence identity with other BVMOs suggests that *in vivo* EtaA is functioning as a BVMO. The kinetic analyses have shown that the observed low turnover rates of the isolated enzyme are because of a relatively low reduction of EtaA by NADPH. It was found that this poor coenzyme reactivity can be significantly increased by adding bovine serum albumin. This indicates that the activity of EtaA in *M. tuberculosis* is modulated by interaction with other cellular components.

Although being a BVMO EtaA accepts ketones as substrate, the exact nature of the physiological substrate(s) remains to be established. Most BVMOs identified so far are involved in the degradation of ketones ranging from small growth substrates like acetol (28) to relatively bulky ketones like cyclododecanone (29). Recent studies have revealed that these bacterial BVMO genes typically are part of an operon, which typically includes a esterase gene to hydrolyze the ester formed by the respective BVMO. However, *etaA* is not part of an operon as it has been found to be directly and singly regulated by a neighboring regulator gene *ethR* (4).

All of the mycobacterial genomes sequenced so far contain an *etaA* gene ortholog showing sequence identity of 100% (*M. tuberculosis* CDC1551 and *Mycobacterium bovis*), 85% (*Mycobacterium marinum*), 81% (*Mycobacterium ulcerans*), 80% (*M. leprae*), 67% (*Mycobacterium paratuberculosis*), 67% (*Mycobacterium avium*), and 65% (*M. smegmatis*). Also, the associated regulator gene *ethR* is conserved in mycobacteria (4). Interestingly, from the six BVMO genes identified in *M. tuberculosis,* only the *etaA* ortholog (80% sequence identity) can be identified in *M. leprae*. Apparently, EtaA is the only BVMO that has survived in *M. leprae*, reflecting the drastic reduction in genome size of this human pathogen (6). Preservation of the *etaA* gene in all of the sequenced mycobacterial genomes suggests that this BVMO serves an important physiological role in mycobacteria. However, EtaA does not seem to be of vital importance for the bacterium because it has been shown that ethionamide-resistant *M. tuberculosis* isolates harbor *etaA* gene mutations, preventing the production of functional EtaA (3). Nevertheless, malfunctioning of EtaA might be rescued to some extent by one or more of the other BVMOs because BVMOs typically display promiscuity in substrate specificity (18).

Because most microbial genomes are lacking BVMO genes, the identification of six BVMOs in the genome of *M. tuberculosis* is striking. Genome analysis of partially and fully sequenced mycobacterial genomes using the BVMO sequence motif reveals that mycobacteria typically contain a relatively large number of BVMOs. Most mycobacterial genomes contain roughly five BVMO genes. At this moment, the record has been set by *M. marinum* containing at least 15 BVMO genes. In line with this scenario, it has been noted before that *M. tuberculosis* contains a relatively large amount of oxidative enzymes including 20 cytochromes P450s (30). The reason for this abundance of oxidative potential in mycobacteria is as yet unknown. It has been suggested that activity of a large number of oxidizing enzymes in *M. tuberculosis* might help the pathogenic organism to survive oxidative stress conditions as encountered in the human macrophage (3). In this context, it can be envisaged that EtaA and/or other mycobacterial BVMOs can play a role in the removal of toxic ketones. The observed exceptional broad substrate specificity of EtaA is in line with fulfilling such a detoxifying function. However, all of the BVMOs characterized so far have been shown to be involved in a specific metabolic process. Because the *etaA* gene is conserved in other mycobacteria, EtaA might also serve a role in a metabolic pathway that is specific for mycobacteria. Typical components of the mycobacterial cell wall are mycolic acids. It has been shown that the ability of mycobacteria to synthesize the so-called wax ester mycolic acids relies on a Baeyer-Villiger oxidation step (31, 32). The BVMO responsible for the synthesis of these wax ester mycolic esters has never been identified. Recently, it has also been suggested that a key step in the degradation of mycolic acids is catalyzed by a BVMO (33). The ability of EtaA to convert relatively hydrophobic substrates and its membrane association would fit well with a function in mycolic acid metabolism. Future biochemical studies should clarify the role of EtaA and its homologs in mycobacterial physiology.

BVMOs are frequently encountered in genomes of mycobacterial pathogens (14, 34, 35). It has even been found that a major part of a BVMO gene (accession number L04542)[2] can be used for PCR-based detection of pathogenic *M. avium* isolates (36). With the exception of a prevalence of BVMOs in mycobacteria, BVMO genes have also been found in other pathogenic microorganisms (37, 38). BVMOs typically display a broad substrate specificity while exhibiting high regio- and enantioselectivity (18). Furthermore, no BVMOs have been identified in plants, animals, or human. This indicates that members of this class of monooxygenases are ideally suited to exploit as prodrug activators because no competing activities exist in the host of these intracellular pathogens, although they catalyze a wide range of selective oxidative reactions. Effective treatment of mycobacterial infections using thioamides (*e.g.* ethionamide) has already shown that the BVMO EtaA can indeed serve as an effective prodrug activator. The identification a new substrate class and corresponding reactivity (Baeyer-Villiger oxidation) for this thioamide activator opens new opportunities for developing chemotherapy for treating mycobacterial infections based on the activity of EtaA or one of its homologs.

REFERENCES

1. Dye, C., Williams, B. G., Espinal, M. A., and Raviglione, M. C. (2002) *Science* **295,** 2042–2046
2. WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance (2002) *Antituberculosis Drug Resistance in the World. Report 2: Prevalence and Trends*, World Health Organization, Geneva, Switzerland
3. DeBarber, A. E., Mdluli, K., Bosman, M., Bekker, L. G., and Barry, C. E., III. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 9677–9682
4. Baulard, A. R., Betts, J. C., Engohang-Ndong, J., Quan, S., McAdam, R. A., Brennan, P. J., Locht, C., and Besra, G. S. (2000) *J. Biol. Chem.* **275,** 28326–28331
5. Pattyn, S. R., and Colston, M. J. (1978) *Lepr. Rev.* **49,** 324–326
6. Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R. M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M. A., Rajandream, M. A., Rutherford, K. M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J. R., and Barrell, B. G. (2001) *Nature* **409,** 1007–1011
7. Grosset, J. H., and Cole, S. T. (2001) *Lepr. Rev.* **72,** 429–440
8. Larsen, M. H., Vilcheze, C., Kremer, L., Besra, G. S., Parsons, L., Salfinger, M., Heifets, L., Hazbon, M. H., Alland, D., Sacchettini, J. C., and Jacobs, W. R., Jr. (2002) *Mol. Microbiol.* **46,** 453–466
9. Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992) *Nature* **358,** 591–593
10. Vannelli, T. A., Dykman, A., and Ortiz de Montellano, P. R. (2002) *J. Biol. Chem.* **277,** 12824–12829
11. Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G., and Jacobs, W. R., Jr. (1994) *Science* **263,** 227–230
12. Johnsson, K., King, D. S., and Schultz, P. G. (1995) *J. Am. Chem. Soc.* **117,** 5009–5010
13. Phetsuksiri, B., Baulard, A. R., Cooper, A. M., Minnikin, D. E., Douglas, J. D., Besra, G. S., and Brennan, P. J. (1999) *Antimicrob. Agents Chemother.* **43,** 1042–1051
14. Fraaije, M. W., Kamerbeek, N. M., van Berkel, W. J. H., and Janssen, D. B. (2002) *FEBS Lett.* **518,** 43–47
15. Kamerbeek, N. M., Moonen, M. J., van der Ven, J. G., van Berkel, W. J. H., Fraaije, M. W., and Janssen, D. B. (2001) *Eur. J. Biochem.* **268,** 2547–2557
16. Kamerbeek, N. M., Olsthoorn, A. J. J., Fraaije, M. W., and Janssen D. B. (2003) *Appl. Environ. Microbiol.* **69,** 419–426

[2] The sequence L04542 can be retrieved from the NCBI database (www.ncbi.nlm.nih.gov/).

17. Massey, V. (1990) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., and Zanetti, G., eds) pp. 59–66, Walter de Gruyter, Berlin, Germany
18. Kamerbeek, N. M., Janssen, D. B., van Berkel, W. J. H., and Fraaije, M. W. (2003) *Adv. Synth. Cat.* **345,** 667–678
19. Ryerson, C. C., Ballou, D. P., and Walsh, C. (1982) *Biochemistry* **21,** 2644–2655
20. Duetz, W. A., van Beilen, J. B., and Witholt, B. (2001) *Curr. Opin. Biotechnol.* **12,** 419–425
21. Meyer, A., Schmid, A., Held, M., Westphal, A. H., Rothlisberger, M., Kohler, H. P., van Berkel, W. J. H., and Witholt, B. (2002) *J. Biol. Chem.* **277,** 5575–5582
22. Sheng, D., Ballou, D. P., and Massey, V. (2001) *Biochemistry* **40,** 11156–11167
23. Einarsdottir, G. H., Stankovich, M. T., Powlowski, J., Ballou, D. P., and Massey, V. (1989) *Biochemistry* **28,** 4161–4168
24. Poulsen, L. L., and Ziegler, D. M. (1995) *Chem. Biol. Interact.* **96,** 57–73
25. Suh, K., Poulsen, L. L., Ziegler, D. M., and Robertus, J. D. (2000) *Arch. Biochem. Biophys.* **381,** 317–322
26. Britton, L. N., and Markovetz, A. J. (1977) *J. Biol. Chem.* **252,** 8561–8566
27. Chakraborty, S., and Massey, V. (2002) *J. Biol. Chem.* **277,** 41507–41516
28. Hartmans, S., and de Bont, J. A. M. (1986) *FEMS Microbiol. Lett.* **36,** 155–158
29. Kostichka, K., Thomas, S. M., Gibson, K. J., Nagarajan, V., and Cheng, Q. (2001) *J. Bacteriol.* **183,** 6478–6486
30. Souter, A., McLean, K. J., Smith, W. E., and Munro, A. W. (2000) *J. Chem. Technol. Biotechnol.* **75,** 933–941
31. Lanéelle, M. A., and Lanéelle, G. (1970) *Eur. J. Biochem.* **12,** 296–305
32. Toriyama, S., Imaizumi, G., Tomiyasi, T., Masui, M., and Yano, I. (1982) *Biochim. Biophys. Acta* **712,** 427–429
33. Asselineau, C., Asselineau, J., Laneelle, G., and Laneelle, M. A. (2002) *Prog. Lipid Res.* **41,** 501–523
34. Howard, S. T., Byrd, T. F., and Lyons, C. R. (2002) *Microbiology* **148,** 2987–2996
35. Rivera-Marrero, C. A., Burroughs, M. A., Masse, R. A., Vannberg, F. O., Leimbach, D. L., Roman, J., and Murtagh, J. J., Jr. (1998) *Microb. Pathog.* **25,** 307–316
36. Thierry, D., Vincent, V., Clement, F., and Guesdon, J. L. (1993) *J. Clin. Microbiol.* **31,** 1048–1054
37. Liang, X., Pham, X. Q., Olson, M. V., and Lory, S. J. (2001) *Bacteriology* **183,** 843–853
38. Yu, J., Chang, P. K., Bhatnagar, D., and Cleveland, T. E. (2001) *Appl. Microbiol. Biotechnol.* **53,** 583–590